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Measurement of Paclitaxel and Its Metabolites in Human Plasma Using a Liquid Chromatography – Ion Trap Mass Spectrometer with a Sonic Spray Ionization Interface

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Running title: Measurement of Paclitaxel Using LC/SSI/Ion Trap

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Abstract

A quantitative liquid chromatography ion trap MS method for the simultaneous determination of paclitaxel, 6α-hydroxypaclitaxel and p-3'-hydroxypaclitaxel in human plasma has been developed and validated. 6α-p-3'-dihydroxypaclitaxel was also quantified using paclitaxel as a reference and docetaxel as an internal standard. The substances were extracted from 0.500 mL plasma using solid phase extraction. The elution was performed with acetonitrile and the samples were reconstituted in the mobile phase. Isocratic HPLC analysis was performed by injecting 50 μL of reconstituted material onto a 100x3.00 mm C12 column with a methanol:1% trifluoroacetic acid/ammonium trifluoroacetate in H2O 70:30 mobile phase at 350 μL/min. The [M+H]+ ions generated in the SSI interface were isolated and fragmented using two serial MS methods: one for paclitaxel (transition 854->569 & 551) and the dihydroxymetabolite (transition 886->585 & 567) and one for the hydroxymetabolites (transition 870->585 & 567; transition 870->569 & 551) and docetaxel ([M+Na]++, transition 830->550). Calibration curves were created ranging between 0.5 and 7500 ng/mL for paclitaxel, 0.5 and 750 ng/mL for 6α-hydroxypaclitaxel and 0.5 and 400 ng/mL for p-3'-hydroxypaclitaxel. Adduct ion formation was noted and investigated during method development and controlled by mobile phase optimization. In conclusion, a sensitive method for simultaneous quantification of paclitaxel and its metabolites suitable for analysis in clinical studies was obtained.
Introduction

Paclitaxel (Taxol®) is an anticancer drug with a broad spectrum of antitumor activity, including breast, ovarian, skin and lung cancer.\(^1\) It was originally isolated from the Pacific yew tree Taxus Brevifolia,\(^2\) and together with the drug docetaxel represents the antineoplastic taxanes. The taxanes have a unique mechanism of action among chemotherapeutic agents in that the drugs facilitate the formation and suppress the depolymerization of microtubules. Exposed cells are blocked in the G2/M phase in the cell cycle and eventually undergo apoptosis.\(^3,\)\(^4\) Paclitaxel also induces apoptosis directly, but this may be a result of different mechanisms at different drug concentrations.\(^5\)

Paclitaxel is primarily metabolized in the liver by the cytochrome P450 (CYP) enzymes, CYP3A4 and CYP2C8. CYP3A4 metabolizes paclitaxel to \(p\)-\(3'\)-hydroxypaclitaxel and CYP2C8 converts the drug to \(6\alpha\)-hydroxypaclitaxel and these metabolites can be further oxidized to \(6\alpha\)-\(p\)-\(3'\)-dihydroxypaclitaxel.\(^6\) Other metabolites have been identified in smaller quantities.\(^7\) Studies have shown that the major portion of unchanged paclitaxel and its metabolites are excreted in the feces, indicating extensive nonrenal clearance.\(^8\) However, only paclitaxel has been shown to be toxic and to have an antitumor activity while its metabolites are considered to be inactive.\(^9\)

Since the beginning of the 1990s, when Taxol was introduced as an anticancer drug,\(^10\) an extensive number of pharmacokinetic studies have been done on the substance. These studies have resulted in the development of several different methods for quantification of paclitaxel in biological samples. The concentrations of paclitaxel and its metabolites in human plasma are measured preferably by HPLC or LC-MS\(^11\), although other techniques have also been
reported. Immunological methods such as enzyme-linked immunoassay (ELISA)\textsuperscript{12} and radioimmunoassay\textsuperscript{13} have been used for screening human plasma, but these methods have a major drawback of cross reactions. Most frequently used are HPLC systems with UV-detection, although other chromatography techniques, e.g. capillary electrophoresis\textsuperscript{14} and methods using other detectors, e.g. fluorescence detectors,\textsuperscript{15} have also been reported.

Several developments in the use of paclitaxel have led to lower plasma concentrations of the drug and the need for more sensitive assays. First of all, clinicians have begun to investigate weekly therapy as an alternative to the every-three-week administration schedule, with a reduction of dose as a result.\textsuperscript{16} In the development of new chemotherapeutic regimens where paclitaxel is used in combination with other drugs, the dose is often less than the recommended phase II dose. In addition, the investigation of individualization of chemotherapy has also led to the need for more accurate techniques. The relative lack of sensitivity and specificity of the HPLC methods with UV-detection\textsuperscript{17} has led to the development of methods using mass spectrometry and multiple MS for detection of the substances.\textsuperscript{17-31} Recent reports have described paclitaxel quantifications based on LC-MS-MS instrumentation. Several different extraction methods, mobile phases, columns, interfaces and MS instruments have been used. However, most of these methods were developed for paclitaxel only and not for the metabolites. For our purposes of trying to predict cancer patient’s individual elimination of paclitaxel by investigating factors affecting the extent of conversion by each metabolic enzyme, we needed an accurate and sensitive method for quantification of paclitaxel and its metabolites in human plasma.

The aim of our study was to develop an LC-MS method for simultaneous quantification of paclitaxel and its metabolites in human plasma. To our knowledge, this is the first method for
quantification of paclitaxel, 6α-hydroxypaclitaxel, p-3'-hydroxypaclitaxel and p-3’-6α-dihydroxypaclitaxel in human plasma using an ion trap mass spectrometer with a sonic spray ionization interface.

**Material and Methods**

**Material**

Paclitaxel (C\(_{47}H_{51}NO_{14}\)) was kindly supplied by Bristol-Myers Squibb (Wallingford, CT, USA). p-3'-hydroxypaclitaxel (C\(_{47}H_{51}NO_{15}\)) and 6α-hydroxypaclitaxel (C\(_{47}H_{51}NO_{15}\)) were purchased from Gentest (Woburn, MA, USA) through BD Biosciences. Docetaxel (C\(_{43}H_{53}NO_{14}\)) was kindly supplied by Aventis Pharma (Vitry Alforville, France) and used as an internal standard. HPLC-grade methanol, acetonitrile and hexane were from LabScan Analytical Sciences (Dublin, Ireland). Ethanol was from Kemetyl (Stockholm, Sweden). Water was obtained from a Milli-Q Biocell station, Millipore AB (Stockholm, Sweden). Trifluoracetic acid was from Sigma (Stockholm, Sweden). Human plasma was delivered from the blood bank of Linköping University Hospital.

**Instrumentation**

The HPLC system consisted of a LaChrome chromatograph (pump model L-7100, autosampler L-7200, UV detector L-7400 and an interface model D-7000) with a Merck-Hitachi M-8000 LC/MS\(^{S}\) (Ion Trap) equipped with a Sonic Spray Ionization (SSI) interface from Merck KgaA (Darmstadt, Germany). The system was controlled using a computer equipped with the Chromatography Data Station Software LC/3DQ MS System Manager from Merck (Darmstadt, Germany) and Hitachi Instruments Inc. (San Jose, CA, USA). An infusion pump from Harvard Apparatus (Holliston, MA, USA), pump 11, was connected to the system during some steps in the optimization. The analytical column was a Synergi 4µ
Measurement of Paclitaxel Using LC/SSSI/Ion Trap

Max-RP 80A, 100x3.00 mm, from Phenomenex (CA, USA) and a guard column, 4x3.0 mm, with the same stationary phase, was used.

Preparation of stock solutions and plasma samples

Paclitaxel was dissolved in methanol to a concentration of 20 mg/mL, and further diluted in methanol to give stock solutions in the range of 0.2-200 μg/mL using glass pipettes. The internal standard docetaxel was dissolved in ethanol to a concentration of 20 mg/mL and diluted to 20 μg/mL. p-3'-hydroxypaclitaxel and 6α-hydroxypaclitaxel were dissolved in acetonitrile and methanol, respectively, to give approximate concentrations of 20 μg/mL. Due to the high inaccuracy of the manufacturer’s quantity determination, the concentrations were determined using absorption spectroscopy. The absorbance was measured at 227 nm for paclitaxel and 230 nm for the other substances. The following molar extinction coefficient were used: 29.8 mM⁻¹cm⁻¹ for paclitaxel, 14.8 mM⁻¹cm⁻¹ for docetaxel, 35.8 mM⁻¹cm⁻¹ for p-3'-hydroxypaclitaxel and 26.2 mM⁻¹cm⁻¹ for 6α-hydroxypaclitaxel (according to the manufacturer’s documentation).

Standards were prepared by diluting the stock solutions in human drug-free plasma. Nine different standards (denoted STD1-STD9) were prepared with the following concentrations 0.5, 2, 5, 10, 50, 250, 1000, 4000, and 7500 ng/mL of paclitaxel, 0.5, 2, 5, 10, 25, 50, 100, 250, and 750 ng/mL of 6α-hydroxypaclitaxel and 0.5, 2, 5, 10, 25, 50, 100, 200, and 400 ng/mL of p-3'-hydroxypaclitaxel. The standard concentrations were based on the concentrations found in human plasma after a dose of 175 mg/m² paclitaxel infused during 3 h.
Five different quality control samples (denoted QC1-QC5) were prepared with the following concentrations: 8, 100, 300, 1500, and 5000 ng/mL of paclitaxel, 8, 20, 40, 80, and 400 ng/mL of 6α-hydroxypaclitaxel and 8, 20, 40, 80, and 300 ng/mL of p-3'-hydroxypaclitaxel.

**Solid phase extraction**

The solid phase extraction (SPE) was adopted from Huizing et al., 1995, with modifications. Prior to extraction, the tips in the vacuum box were washed in acetonitrile to minimize crossover. The CN-solid phase (100 mg, 1 mL, Isolute, IST U.K.) columns were conditioned with 2 mL methanol and 2 mL 10 mM ammonium acetate buffer, pH 5.0. 500 µL of spiked plasma were mixed with an equal volume of 0.2 M ammonium acetate, pH 5.0, and 50 µL of internal standard (20 µg/mL) and applied to the column. The columns were washed with 2 mL of 10 mM ammonium acetate and 1 mL of methanol:10 mM ammonium acetate, 20:80, and finally with 1 mL of hexane. Prior to and after the hexane wash, the columns were dried for a minimum of 2 minutes. The samples were eluted in 2 mL of acetonitrile and evaporated under a nitrogen stream at 35°C. The analytes were reconstituted in 200 µL mobile phase and sonicated for 5 minutes to ensure that the compounds dissolved completely. The samples were then placed in the autosampler and 50 µL were injected twice from each vial.

**LC/MS optimization and settings**

Several mobile phases were tested to yield a sensitive and stable chromatography and MS detection. The mobile phase finally used for chromatographic separation was methanol:1% trifluoroacetic acid/ammonium trifluoroacetate in H₂O, pH 7, 70:30 with a flow of 350 µL/min and the chromatography ran for 9 minutes.
The SSI settings were as follows: plate temperature 200°C, aperture 1 temperature 150°C, aperture 2 temperature 120 °C, chamber voltage 0.6 kV, drift voltage 35 V and focus voltage 34 V.

In the Ion Trap Mass Spectrometer positive ion polarity was used, the multiplier voltage being set to 400 V and the peak threshold to 10. The Automatic Sensitivity Control (ASC) was turned on and, for each run, three microscans were centroided and averaged. The mass spectrometer was run in \( \text{MS}^2 \) mode using two alternating methods. The first \( \text{MS}^2 \) method was used for detection of paclitaxel and dihydroxypaclitaxel (a reference substance was not available for this metabolite so paclitaxel was used as reference). In the ion accumulation step for this method, the low mass cutoff (LMC) was set to 80, the accumulation mass range was set to 750-940 \( m/z \), the accumulation voltage was set to 0.03 V and the sensitivity was 200. The settings for the isolation step were as follows: isolation mass range 849-859 and 881-891, LMC 750, isolation voltage 0.15 V, and isolation time 10 ms. During the collision-induced dissociation (CID) step, the following settings were used: CID resonance 849-863 and 876-896, LMC 420 V, CID time 50 ms, and CID voltage 0.15 V. The second \( \text{MS}^2 \) method was used for the detection of both hydroxypaclitaxel metabolites and docetaxel. The settings for the ion accumulation step were as follows: the accumulation mass range was set to 766-940 \( m/z \), the accumulation voltage was set to 0.03 V, and the sensitivity was 200. During the isolation step, the following settings were used: isolation mass range 825-835 and 865-875, LMC 770, isolation voltage 0.12 V, and isolation time 10 ms. In the CID step, LMC was set to 420, CID resonance to 825-835 and 865-881 \( m/z \), CID time to 50 ms, and CID voltage to 0.2 V. During both methods the MS spectra were scanned for a mass-to-charge ratio between 500 and 600 \( m/z \). Chromatograms for each substance were extracted from the MS chromatograms using the product ions as indicated in Table 1.
Validation procedure

The precision and accuracy of the method were evaluated by analyzing paclitaxel, \( p-3'\)-hydroxypaclitaxel and 6\( \alpha \)-hydroxypaclitaxel at five different concentrations (QC1-QC5) on three different days. Six quality control samples at each concentration were used to evaluate within-day variation and six were used for between-day variation. To determine the concentration of the analytes in the QC-samples one calibration curve consisting of nine different concentrations (STD1-STD9) were analyzed each day. Calibration was performed using the area of the corresponding peak in the chromatogram and the calibration curve was created using a cubic-fit regression model forced through zero.

The precision of the assay was expressed as a coefficient of variation (CV) at each concentration by calculating the standard deviation as a percentage of the mean calculated concentrations, while accuracy of the method was evaluated by expressing the mean calculated concentration as a percentage of the nominal concentration. The percentage extraction recoveries of paclitaxel, \( p-3'\)-hydroxypaclitaxel and 6\( \alpha \)-hydroxypaclitaxel for the quality control samples were determined by comparing extracted blank samples in which the SPE eluate had been spiked with a corresponding amount of substance with those obtained by injections of extracted spiked plasma samples. The stability of paclitaxel, \( p-3'\)-hydroxypaclitaxel and 6\( \alpha \)-hydroxypaclitaxel in the samples was checked in three different experiments; one for room temperature stability, one for stability after reconstitution in the mobile phase and one experiment for freeze-thaw stability. The stability at room temperature was evaluated by letting eight samples of spiked plasma at two different concentrations (paclitaxel 50 and 1000 ng/mL, \( p-3'\)-hydroxypaclitaxel 50 and 200 ng/mL, and 6\( \alpha \)-hydroxypaclitaxel 50 and 200 ng/mL) stand at room temperature for 24 h and another set of
Measurement of Paclitaxel Using LC/SSI/Ion Trap

eight samples for 48 h. The concentrations were then compared with the quality control samples with corresponding concentrations. To check the stability after reconstitution, ten QC-samples were left on the bench after injection and re-injected four days later. The areas of the peaks were then compared to the first injection. Eight plasma samples, at the same two concentrations as for the room temperature stability test, were frozen to -70°C and thawed for three cycles and then extracted and analyzed. The areas of the peaks were compared to the quality control samples spiked with the same concentrations.

Results and Discussion

Method Development

The major goal in the development of this assay was to quantify low concentrations of both paclitaxel and its hydroxy metabolites in human plasma. Due to the fact that we only had access to small amounts of the metabolites, the method was first developed for paclitaxel and then the settings were adopted to fit the quantification of the available metabolites and thereafter fine-tuned for the best performance.

In most instruments and previously reported methods, paclitaxel either forms the [M+H]⁺ ion or the [M+Na]⁺ molecular ion. To suppress the formation of the Na⁺ adduct in favor of the [M+H]⁺ ion an acidic mobile phase would be required. However, in our system we could not get a reproducible formation of the [M+H]⁺ ion between different batches of acidic mobile phase due to the formation of a lower or higher amount of [M+Na]⁺. To stabilize the system the mobile phase was changed to push paclitaxel to form other ions instead of the [M+H]⁺. By adding positive or negative ions to the mobile phase, the following adducts could be detected in the LC/MS: [M+K]⁺ (2 mM KCl:MeOH 30:70), [M+Na]⁺ (2 mM NaCl:MeOH 30:70), and
[M+Acetate] (20 mM ammoniumacetate pH 5.0:MeOH 30:70). The highest sensitivity of these adducts was reached with the [M+K]+ ion. However, the [M+H]+ ion showed a higher sensitivity, although unstable, than any of the other ions, probably due to fragmentation of the latter in the ion source. We then tried to stabilize the formation of the [M+H]+ ion by suppressing the [M+Na]+ ion. This was done using crown ethers or trifluoroacetic acid (TFA), the later giving the highest sensitivity and the lowest amount of [M+Na]+ adducts. Although TFA caused a suppression of the absolute signal, the signal-to-noise ratio for paclitaxel was increased and a stable [M+H]+ ion formation was achieved. The mobile phase was then optimized for TFA concentration and pH. Optimum MS² conditions for the analyte response were established using a continuous infusion of a stock solution containing paclitaxel while running the chosen mobile phase components. Note that docetaxel forms the [M+Na]+ adduct using this mobile phase and instrument.

Several interfaces were also tested to evaluate the response. The M-8000 LC/MS⁰ Ion Trap was equipped with both an SSI interface as well as an Electro Spray Ionization (ESI) interface. The SSI interface was about 4 times more efficient in ionizing paclitaxel than the ESI. We also had the opportunity to test the ionization on a Perkin Elmer SCIEX equipped with both an ESI and an Atmospheric Pressure Chemical Ionization (APCI) interface. In this system, the ESI was more efficient in ionizing paclitaxel than the APCI interface. The SSI interface was then considered the best choice.

The HPLC column was chosen to retain the analytes sufficiently to avoid ion suppression by early eluting substances in the sample extract and separate the analytes since some of them have identical mass-to-charge ratios for some fragments. The monohydroxymetabolites have the same molecular mass but they fragment into ions with a different m/z. However, p-3´-
hydroxypaclitaxel dissociate into fragments with the same \( m/z \) as fragments from paclitaxel and 6\( \alpha \)-hydroxypaclitaxel dissociates into fragments with the same \( m/z \) as dihydroxypaclitaxel (Figure 1). To achieve single-peak mass chromatograms two methods were run in parallel, one for paclitaxel and dihydroxypaclitaxel and one for the monohydroxymetabolites (Table 1). By making sure that the HPLC conditions yielded peak separation, interference between the analytes was minimized. Typical retention times were 6.05 min for paclitaxel, 3.65 min for \( p \)-3\( \alpha \)-hydroxypaclitaxel, 5.0 min for 6\( \alpha \)-hydroxypaclitaxel, 3.3 min for dihydroxypaclitaxel, and 6.8 min for docetaxel. After each injection the chromatography was run for 9 min. Figure 2 shows typical chromatograms for paclitaxel and its hydroxylated metabolites generated from the product ions formed in MS\(^2\) (\( m/z \) shown in Table 1). Docetaxel was used as an internal standard, however, it did not increase the precision or accuracy of the method and was therefore only used to control the SPE and autosampler.

Paclitaxel adheres strongly to most surfaces; carry-over effects and ghost peaks were major problems during early method development. The problems were traced to the SPE and solved by replacing the nylon tips in the SPE vacuum box with steel needles, which were washed between all extractions.

Each sample was injected twice due to spiking of the chromatograms. We were not able to get rid of the spiking phenomenon by adjusting the MS conditions or cleaning of the MS, although improving the water quality in the mobile phase helped some. The spiking was, however, rather infrequent, and readily identified, and if one of the chromatograms contained interference, the other injection was used. With more advanced software, it would be rather easy just to exclude a single data point caused by spiking instead of having to run the samples twice.
Due to the wide range of paclitaxel concentrations found in plasma, the calibration curve for paclitaxel was divided into a low (0.5-250 ng/mL) and high-range (250-7500 ng/mL) calibration curve. The calibration curve was also created using a cubic-fit regression model due to nonlinearity at high paclitaxel concentrations. The response is linear from 0.5 ng/mL up to 1000 ng/mL. Proposed causes of non-linearity at the high end of the calibration curve have been the formation of paclitaxel dimers and trimers at the interface as well as charge transfer in the trap at high ion concentrations. Others have solved this nonlinearity by diluting the samples. However, we found that in the range presented in this assay, the use of nonlinear calibration curves worked just as well. The calibration curves for the metabolites were linear over the whole calibration range. Figure 3 shows a calibration curve for paclitaxel obtained during one of the validation runs.

The final method conditions were chosen for several reasons. First of all they allow the quantification of low concentrations (<10 ng/mL) of the analytes in human plasma. Second, they allow the determination of paclitaxel and its metabolites simultaneously. The method presented here has some special features, especially the use of TFA as a Na-adduct suppressor and the SSI interface, both of which improved the performance of the assay.

_Precision and Accuracy_

The reproducibility of the method was evaluated by analyzing six replicates of five different QC samples on three different days. Extracted standard curves obtained in the range of 0.5 – 7500 ng/mL showed excellent curve fitting with a coefficient of correlation greater than 0.99. The within-day and between-day variations are reported in Table 2. The method was found to be acceptably precise (CV < 14 %) and accurate (range 83-113%, except for the within-day
variation of paclitaxel at the lowest concentration). The mean extraction recovery, assessed at the same concentrations as the QC samples, was a bit low_ paclitaxel range 63-74%, p-3′-hydroxypaclitaxel 65-71%, and 6α-hydroxypaclitaxel 72-85%. The quantification of paclitaxel and its hydroxymetabolites was possible within the range of the standard curves. The lower limit of quantification was set to 0.5 ng/mL for paclitaxel (within-day precision <12%, n=4, and between-day accuracy 103%-122%, n=3) and to 2 ng/mL for the hydroxymetabolites (within day variation <20%, n=4, and between-day accuracy 107%-125%, n=3).

Stability Experiments

The results of the stability tests on the samples are presented in Tables 2 and 3. Freeze-thaw cycling did not affect the precision or accuracy of the assay. The substances in plasma were also stable at room temperature for at least 48 h and after reconstitution in the mobile phase, the substances were stable for at least 4 days.

General Discussion

Several mass spectrometry methods have been developed for the quantification of paclitaxel in different matrixes. In most methods the [M+H]+ ion has been used for MS analysis, which we found to have the highest sensitivity, although other adducts such as [M+NH4]+, [M+Na]+, [M+M+Na]+, and [M+Acetate]− have been found. In the early characterizations of paclitaxel and its metabolites, fast atom bombardment was used for ionization of the compounds. Most modern methods use the ESI interface, although the APCI and now the SSI have been shown to be useful. Some of the more impressive methods developed are those published by Schellen et al., 2000, which has an analysis time of only 80 s, and by Parise et al., 2003, which has a range of almost 4 decades. Although
many methods have been published for paclitaxel, only a few MS methods have been
developed for the quantification of the metabolites. Royer et al., 1995, used a MS\(^1\)
method with an APCI interface and Mortier et al., 2005, and Alexander et al., 2003,
used MS\(^2\) methods on triple quadrupole instrument with ESI interfaces as compared to our use of
ion trap methodology and a SSI interface. Mortier et al. developed a method for quantification
in oral fluids and human plasma with a liquid-liquid extraction (LLE) and gradient
chromatography. The calibration ranges in plasma were 4-100 ng/mL for paclitaxel and 2-100
ng/mL for the metabolites. The method developed by Alexander et al. is used for
quantification of the substances in dog and human plasma. LLE was used for sample
preparation and \(^{13}\)C\(_6\)-labeled paclitaxel was used as an internal standard. The calibration
ranges in plasma were 0.1 – 100 ng/mL for all compounds. We used a SPE instead of LLE to
minimize ion suppression (we also had difficulties with the recovery of the metabolites when
using LLE) by unwanted compounds, hopefully giving us a more robust method, and isocratic
chromatography since the mobile phase was shown to be of such importance for stable ion
formation. Our assay also has a rather wide calibration range of 0.5-7500 ng/mL for
paclitaxel, 0.5-750 ng/mL for 6\(\alpha\)-hydroxypaclitaxel, and 0.5-400 ng/mL for \(p\)-3'-
hydroxypaclitaxel, which were chosen according to the concentrations found in human
plasma after a 3-h infusion of 175 mg/m\(^2\) Taxol\(\textregistered\). To our knowledge the use of TFA as a
stabilizer of [M+H]\(^+\) formation and an increase in signal to noise ratio has not been presented
before and the effect is probably due to the Na\(^+\) binding effect of TFA. The responses of the
metabolites were linear but in the case of paclitaxel a cubic-fit regression model was used due
to nonlinearity. Others have also found this nonlinearity and solved the problem by either
diluting the sample or using nonlinear regression.
Conclusion

This is the first reported chromatographic method quantifying paclitaxel and its metabolites in human plasma using LC-SSI-Ion Trap methodology. Comprehensive method development with optimization of the mobile phase to yield as stable and marked formation of the [M+H]^+ ion as possible led to an assay with a high response. The development of a highly sensitive method with a wide calibration range for simultaneous quantification of paclitaxel and its metabolites in human plasma was a prerequisite for this project. The validation was performed successfully. The advantages of this method are the high sensitivity, the simultaneous quantification of all compounds and the use of a relatively low cost SSI/Ion Trap instrument compared with triple quadrupole mass spectrometer; the drawback is the spiking of the chromatograms, which may call for reanalysis of single samples. This analytical method has been used to quantify paclitaxel and its metabolites in more than 200 plasma samples from patients with ovarian cancer and will be used in the future to evaluate the conditions for individualized chemotherapy with paclitaxel.

Acknowledgment

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### Table 1. Product ions and isolation m/z in the MS and retention time (Rt) for the analytes.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Rt (min)</th>
<th>Isolation m/z</th>
<th>Product ions</th>
<th>Method #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>6.1</td>
<td>849-859</td>
<td>550+551+568+569</td>
<td>1</td>
</tr>
<tr>
<td>p-3'-hydroxypaclitaxel</td>
<td>3.6</td>
<td>865-875</td>
<td>550+551+568+569</td>
<td>2</td>
</tr>
<tr>
<td>6α-hydroxypaclitaxel</td>
<td>5.1</td>
<td>865-875</td>
<td>566+567+583+586</td>
<td>2</td>
</tr>
<tr>
<td>6α-,p-3'-dihydroxypaclitaxel</td>
<td>3</td>
<td>881-891</td>
<td>566+567+583+586</td>
<td>1</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>7</td>
<td>825-835</td>
<td>548-551</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Precision and accuracy of the quantification of paclitaxel and its monohydroxy metabolites in plasma. The last column represents the stability of the substances after reconstitution in the mobile phase.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Sample⁸ Concentration ng/mL plasma</th>
<th>Accuracy Within-day mean</th>
<th>Between-day mean</th>
<th>Precision Within-day CV</th>
<th>Between-day CV</th>
<th>Re-inj. 4 days later mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>QS1 (n=6)</td>
<td>8</td>
<td>151%</td>
<td>93%</td>
<td>4%</td>
<td>13%</td>
</tr>
<tr>
<td></td>
<td>QS2 (n=6)</td>
<td>100</td>
<td>84%</td>
<td>91%</td>
<td>9%</td>
<td>6%</td>
</tr>
<tr>
<td></td>
<td>QS3 (n=6)</td>
<td>300</td>
<td>87%</td>
<td>100%</td>
<td>7%</td>
<td>8%</td>
</tr>
<tr>
<td></td>
<td>QS4 (n=6)</td>
<td>1500</td>
<td>96%</td>
<td>100%</td>
<td>4%</td>
<td>4%</td>
</tr>
<tr>
<td></td>
<td>QS5 (n=6)</td>
<td>5000</td>
<td>92%</td>
<td>87%</td>
<td>7%</td>
<td>7%</td>
</tr>
<tr>
<td>6-α-OH-Pac</td>
<td>QS1 (n=6)</td>
<td>8</td>
<td>106%</td>
<td>95%</td>
<td>2%</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>QS2 (n=6)</td>
<td>20</td>
<td>108%</td>
<td>97%</td>
<td>6%</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>QS3 (n=6)</td>
<td>40</td>
<td>95%</td>
<td>83%</td>
<td>9%</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>QS4 (n=6)</td>
<td>80</td>
<td>86%</td>
<td>84%</td>
<td>7%</td>
<td>4%</td>
</tr>
<tr>
<td></td>
<td>QS5 (n=6)</td>
<td>400</td>
<td>93%</td>
<td>97%</td>
<td>9%</td>
<td>14%</td>
</tr>
<tr>
<td>p-3'-OH-Pac</td>
<td>QS1 (n=6)</td>
<td>8</td>
<td>113%</td>
<td>101%</td>
<td>9%</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>QS2 (n=6)</td>
<td>20</td>
<td>113%</td>
<td>99%</td>
<td>7%</td>
<td>12%</td>
</tr>
<tr>
<td></td>
<td>QS3 (n=6)</td>
<td>40</td>
<td>105%</td>
<td>90%</td>
<td>9%</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>QS4 (n=6)</td>
<td>80</td>
<td>102%</td>
<td>93%</td>
<td>3%</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>QS5 (n=6)</td>
<td>300</td>
<td>103%</td>
<td>92%</td>
<td>9%</td>
<td>11%</td>
</tr>
</tbody>
</table>

Notes: ¹ The within-day accuracy on the following three days were 80%, 103% and 88%. ⁸ For each concentration six samples were tested for the within-day variation, for the between-day variation two samples on three different days (n=6) were used for each concentration and the re-injection was done on the within-day variation samples.
Table 3. The stability of paclitaxel, p-3′-hydroxypaclitaxel and 6α-hydroxypaclitaxel during three cycles of freeze-thawing to -70°C and room temperature (RT) stability for 24h and 48h.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Conc. ng/mL plasma</th>
<th>Freeze-thawing mean</th>
<th>RT 24 h mean</th>
<th>RT 48 h mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>1000 (n=4)</td>
<td>103%</td>
<td>97%</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td>50 (n=4)</td>
<td>117%</td>
<td>110%</td>
<td>116%</td>
</tr>
<tr>
<td>6α-OH-pac</td>
<td>200 (n=4)</td>
<td>89%</td>
<td>96%</td>
<td>91%</td>
</tr>
<tr>
<td></td>
<td>50 (n=4)</td>
<td>117%</td>
<td>112%</td>
<td>114%</td>
</tr>
<tr>
<td>p-3′-OH-pac</td>
<td>200 (n=4)</td>
<td>116%</td>
<td>104%</td>
<td>109%</td>
</tr>
<tr>
<td></td>
<td>50 (n=4)</td>
<td>115%</td>
<td>105%</td>
<td>101%</td>
</tr>
</tbody>
</table>
**Figure legends**

**Figure 1.** Molecular structure and fragmentation of paclitaxel and its monohydroxy metabolites.

**Figure 2.** Typical chromatograms for paclitaxel and its hydroxylated metabolites isolated from plasma (15 minutes post end of infusion) from a patient with ovarian cancer receiving 175 mg/m² of paclitaxel (Taxol®). The chromatograms were generated using the sum of the product ions formed in MS² (550+551+568+569 m/z for paclitaxel, 550+551+568+569 m/z for p-3’-hydroxy- paclitaxel, and 566+567+583-586 m/z for 6α-hydroxypaclitaxel). In the chromatogram the baseline for the 6α-hydroxypaclitaxel is elevated with 5000 AU and that for paclitaxel with 100,000 AU for illustrative purposes. In this method the Ion Trap works with two serial methods: one generating the paclitaxel (and the dihydroxymetabolite) chromatogram and one the hydroxymetabolites (and the internal standard). Note that the peak at 6.1 min in the p-3’-hydroxy- paclitaxel chromatogram is from paclitaxel (more than 100-fold intensity in the correct method) and that the peak at 7 min in the 6α-hydroxypaclitaxel chromatogram is from the internal standard, which has a $^{13}$C fragment with the same m/z as the metabolite.

**Figure 3.** A nonlinear calibration curve for the higher range of paclitaxel concentrations obtained during one of the validation runs. The concentration of paclitaxel in spiked standard samples is shown on the x-axis and the response of the MS as the area of the peak is presented on the y-axis. A cubic-fit regression model was used for calibration of the paclitaxel concentrations. The responses of the metabolites were linear within the calibration ranges.
Figure 1

Paclitaxel+1: 854 m/z
OH-Pac+1: 870 m/z

Paclitaxel: 569 m/z
p’3-OH-Pac+1: 569 m/z
6α-OH-Pac+1: 585 m/z

Paclitaxel: 551 m/z
p’3-OH-Pac+1: 551 m/z
6α-OH-Pac+1: 567 m/z
Figure 2

Figure 3